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Yu-Wen Hu

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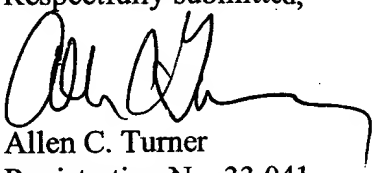
Enclosed is a Certified Copy of the Canadian Priority Application 2,245,039.

Respectfully submitted,



Allen C. Turner
Registration No. 33,041
Attorney for Applicant(s)
TRASKBRITT, PC
P.O. Box 2550
Salt Lake City, Utah 84110-2550
Telephone: 801-532-1922

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Specification and Drawings, as originally filed, with Application for Patent Serial No: **2,245,039**, on August 13, 1998, by **CANADIAN RED CROSS SOCIETY**, assignee of Yu-Wen Hu, Evan Balaskas, Garry Kessler, Claudia Issid, Linda J. Scully, Donald G. Murphy, Aline Rinfret, Cecilia Smeenk, Antonio Giulivi, Vito Scalia and Peter Gill, for "Primer-Specific and Mispair Extension Assay for Identifying Gene Variation".

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PRIMER-SPECIFIC AND MISPAIR EXTENSION ASSAY FOR IDENTIFYING GENE VARIATION

BACKGROUND OF THE INVENTION

(a) Field of the Invention

The invention relates to primer-specific and mispair extension assay for identifying gene variations, such as in different genotypes or subtypes of a given genotype.

(b) Description of Prior Art

Current genotyping systems are technically complex, time-consuming and error-prone in the detection of a single nucleotide variation and low level heterozygotes.

Despite current genotyping systems such as fragment length polymorphism analysis (RFLP)[1], hybridization (e.g. line probe assay, LiPA)[2], selective DNA amplification by PCR-type specific primers (Okamoto, H., et al., *J. Gen. Virol.* **73**:673, 678,1992) and direct DNA sequencing having been useful in general, some technical problems still remain and limits their applications.

For most indirect DNA sequencing genotyping systems, a common weakness is that they are not as accurate as direct DNA sequencing analysis in particular for detection of a single nucleotide mutation or variation, resulting in considerable instances of errors or inconsistent results (Andonov, A., et al., *J. Clin. Microbiol.* **32.** 2031-2034,1994); (Tuveri, R., et al., *Journal of Medical Virology* **51:** 36-41 (1997); Okamoto, H., et al., *J. Virol. Methods* **57**:31 45 002-16, 1996). Although direct DNA sequencing is the most reliable method for genotyping, this is not practical for large cohort studies.

Another major limitation of all current genotyping systems, including direct DNA sequencing is

that they can not reliably detect low level of heterozygotes (Tuveri, R., et al., *Journal of Medical Virology* **51:** 36-41, 1997); (Lau, J.Y., et al., *J. Infect. Dis.* **171:** 281-289, 1995); (Forns, X., et al., *J. Clin. Microbiology.* **34-10:** 2516-2521, 1996); or mixed genotype infectious (Tuveri, R., et al., *Journal of Medical Virology* **51:** 36-41, 1997); (Lau, J.Y., et al., *J. Infect. Dis.* **171:** 281-289, 1995); (Forns, X., et al., *J. Clin. Microbiology.* **34-10:** 2516-2521, 1996).

HCV was recognized as the major etiologic agent of blood borne non-A, non-B hepatitis soon after the virus was identified in 1989. As an RNA virus, HCV shows great genetic variability, resulting in the existence of types, subtypes and quasispecies. At present, 11 types and at least 50 subtypes have been described. However, types 1a, 1b, 2a, 2b and 3a have been found to be generally the most prevalent (Simmonds, P., *Hepatology* **21:** 570-582, 1995). Subtype 1b is the most common genotype found in Japan (Okamoto, H., et al., *J. Gen. Virol.* **73:**673, 678, 1992) and European countries while subtypes 1a and 1b are the most common genotypes in the United States (Lau, J.Y., et al., *J. Infect. Dis.* **171:** 281-289, 1995) and Canada, (Andonov, A., et al., *J. Clin. Microbiol.* **32:** 2031-2034, 1994). Viruses of various genotypes contain different antigenic properties, which have potentially important consequences for the development of a vaccine and for antibody screening tests. Also, the disease severity and response to interferon may be influenced by the virus types and subtypes (Simmonds, *Hepatology* **21:** 570-582, (1995). Subtype 1b was reported to be associated with a high severity of the disease and low response to interferon (Simmonds, *Hepatology* **21:** 570-582, (1995). It is apparent that a rapid, simple,

accurate and inexpensive genotyping method is urgently needed.

Amplification refractory mutation system (ARMS) (Newton, C.R., et al., *Nucl. Acids Res.* **17:**2503-2516), improved the methods used in the prior art for typing the five most common genotypes (Pistello, M., et al., *J. Clin. Microbiol.* **32**: 232-234, 1994). ARMS was developed for PCR detection of any point mutation in DNA using *Taq* DNA polymerase (Newton, C.R., et al., *Nucl. Acids Res.* **17:**2503-2516).. It is based on the principle that oligonucleotides with a mismatched 3'-residue would not function as primers in PCR under controlled conditions. In some cases, however, the specificity of ARMS was not sufficient to give a correct diagnosis. The problem with non-specific reactivities still remains with the type-specific primer PCR method for HCV genotyping, even with the improvement using ARMS.

It is apparent that the major cause of the nonspecific reactivities found in these assays is related to the use of *Taq* DNA polymerase due to its lack of 3'→5' exonuclease activity. This inaccuracy results in base substitutions, transitions, tranversions, frame shifts or deletion mutations during DNA synthesis. Consequently, mispairs can be frequently formed, and *Taq* polymerase would be able to continue synthesizing DNA by addition of the next correct nucleotide on the template (Lau, J.Y., et al., *J. Infect. Dis.* **171:** 281-289, 1995). Even after reaching the end of the template, several more nucleotides can be added to the extended primer because most DNA polymerases, including *Taq* and retroviral reverse transcriptase (RT), have a non-template dependent DNA synthesis activity (i.e. terminal deoxynucleotide transferase activity) (Clark, J.M.,

Nucleic Acids Res. **16:**9677; (Patel, P.H., et al., *Proc. Natl. Acad. Sci. U.S.A.* **91:** 549-553). Therefore, the nonspecific reaction cannot be avoided with either ARMS or the methods based on ARMS using *Taq* DNA polymerase.

Two thermostable DNA polymerases *pfu* (*pyrococcus furiosus*) (Lundberg, K.S., et al., *Pyrococcus furiousus. Gene* **108:** 1-6, 1991), and *TLI/Vent* (*Thermococococcus litoralis*) (Neuner, A., et al., *Iarch. Microbiol.*, **153:**205-207) exhibit 3'→5' proofreading exonuclease activity. This ensures a high degree of amplification fidelity during DNA polymerization. Unlike *Vent*, the *pfu* 3'→5' exonuclease activity peaks sharply at its optimal polymerization temperature (75°C to 80°C), minimizing undesirable primer-degradation activity (Lundberg, K.S., et al., *Gene* **108:** 1-6, 1991). *pfu* DNA polymerase also does not exhibit terminal deoxynucleotidyl-transferase (TDT) activity, which was reported to be involved in the high mutation rate of DNA during DNA polymerization.

It would be highly desirable to be provided with a simple assay or method to overcome many of these limitations of current DNA genotyping systems.

SUMMARY OF THE INVENTION

One aim of the present invention is to provide a novel primer specific and mispair extension assay (PSMEA) for the determination of genotypes and subtypes.

Another aim of the present invention is to provide a primer specific and mispair extension assay (PSMEA) to detect nucleotide variations in any known gene sequence using *pfu* DNA polymerase in the presence of an incomplete set of dNTPs.

Another aim of the present invention is to provide a tool for reliable detection of mixed genotype infectius.

Another aim of the present invention is to provide a tool for accurate genotyping.

In accordance with the present invention there is provided a primer-specific and mispair extension assay for the determination of genotype and detection of low level mixed genotype injections or heterozygotes. The assay comprises the steps of:

a) extending a DNA sequence amplified from a patient sample with *PFU* DNA polymerase using a primer specific for a genotype to be determined and an incomplete set of dNTPs, under suitable conditions for obtaining extension of the primer, whereby at least one of the primer or one of the dNTPs is labelled;

b) separating the extended DNA sequences obtained in step a);and

c) detecting the separated extended DNA sequences; and

d) comparing the extended DNA sequences with known DNA sequences of various genotypes for determining the genotype of the DNA sequences extended.

The primer may be end-labelled with a label or one of the dNTPs can be labelled with a label. The label can be a radioactive or fluorescent label.

Preferably, the steps a), b), c), and d) as described above are automated.

Preferably step c) further comprises the step of sequencing the separated amplified DNA sequences.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 illustrates characteristics of primer specific and mispair extension by *pfu* and Taq. DNA polymerase;

Fig. 2 illustrates nucleotide deletions and insertions by PSMEA with primer 6AR-1 and 6AR-2 extensions by *pfu* on templates 1a and 6a);

Fig. 3 illustrates a comparison of the sensitivity between PSMEA and direct DNA sequencing in detection of mixed genotypes; and

Fig. 4 illustrates a typical profile of unlabelled primer extensions by *pfu* on HCV templates for types 1a, 1b, 2a, 2b, 3a and 3b using ^{32}P-labelled dCTP and dGTP.

DETAILED DESCRIPTION OF THE INVENTION

In accordance with one embodiment of the invention, there is provided a novel primer specific and mispair extension assay (PSMEA) for the determination of genotypes, and more preferably Hepatitis C virus (HCV) genotypes and subtypes.

PSMEA is used to detect nucleotide variations in any known gene sequence using *pfu* DNA polymerase in the presence of an incomplete set of dNTPs. To test the feasibility of PSMEA, the 5' UR of the HCV genome, known for being highly conservative, was used as a model for analysis of the nucleotide variation in determining the type and subtype of the virus. The results obtained demonstrate that PSMEA is a rapid, simple and accurate method for HCV genotyping.

In the 5' UR of the HCV genome, six major genotypes and some subtypes can be classified by the nucleotide variation in this region (Simmonds, P., *Hepatology* **21:** 570-582, 1995); (Stuyver, L.,et al., *J. Clin. Microbiol.* **34:**2259-2266, 1996).

The PSMEA of the present invention is a simple method with great potential in accurately detecting nucleotide mutations and which may be used for

detecting nucleotide variations in any known gene sequence.

PSMEA is based on the unique properties of 3'→5' proofreading activity in a reaction with incomplete set of dNTPs. Under such reaction condition in accordance with one embodiment of the invention, *pfu* is extremely discriminative in nucleotide incorporation and proofreading at the initiation step of DNA synthesis, allowing for an accurate detection of nucleotide variation and heterozygotes in PSMEA.

It is known that under this reaction condition, mispair formation and extension occur during DNA synthesis when using reverse transcriptase and DNA polymerases, including enzymes that exhibit 3'→5' proofreading exonuclease activity (Perinno, F., et al., *Proc. Natl. Acad. Sci. USA.* **86**:8343-8347, 1989); (Reha-Krantz, L.J., et al., *Proc. Natl. Acad. Sci. USA.* **88**:2417-2421, 1991). However, the frequency of mispair formation and extension depends on whether the polymerase possesses 3'→5' exonuclease activity, the concentration of nucleotide substrates and the composition of the mispairs (Reha-Krantz, et al., *Proc. Natl. Acad. Sci. USA.* **88**:2417-2421, 1991). Accordingly, the characteristics of primer specific and mispair extension by thermostable DNA polymerases including *pfu* (*Pyrococcus furiosus*) (Lundberg, K.S., et al., *Gene* **108**: 1-6,(1991), *Taq* (*Thermus aquaticus*), (Saiki, R.K., et al., *Science.* **239**:487, 1988), and *TLI/Vent* (*Thermococococcus litoralis*) (Neuner, A., et al., *Iarch. Microbiol.*, **153**:205-207) were further investigated. Several characteristics of primer specific and mispair extension by *pfu* were observed and found to be useful for reliable detection of nucleotide variation, deletion and insertion as well as heterozygotes.

In accordance with a preferred embodiment of the invention, the accuracy was evaluated by assaying the nucleotide variations between genotypes in the 5' untranslated region (5' UR) of the hepatitis C virus (HCV) genome. Mixed infections with more than one genotype of HCV were used for comparison of the sensitivity of PSMEA with other assays in the detection of heterozygotes. The feasibility of the method of the present invention for large cohort studies was demonstrated by genotyping a total of two hundred and forty five (245) HCV isolates. The results show that PSMEA is an extraordinary accurate system to identify nucleotide variation for genotyping and detecting heterozygous molecules, readily applicable to routine use.

The design of PSMEA in accordance with a preferred embodiment of the invention is based on a single primer extension by the *pfu* DNA polymerase in the presence of only dGTP and dGTP, permitting accurate detection of nucleotide variations in the 5' untranslated region (5'UR) of the HCV genome. In accordance with one embodiment of the invention, the HCV genotypes from ninety-six (96) patients and blood donors with HCV infection were determined by PSMEA. Seventy-four (74) of the samples were also genotyped by either the line probe assay (LiPA) or restriction fragment length polymorphism (RFLP) methods. Genotypes were confirmed by nucleotide sequencing as required. HCV Isolates, including types and subtypes 1a, 1b, 2a, 2b, 2c, 3a, 3b, 4a, 5a, and 6a, were clearly identified by the PSMEA of the present invention. All of the types and subtypes determined by PSMEA were matched with those identified by LiPA or RFLP. Five (5) isolates of subtypes 1a and 1b that could not be typed

by LiPA were clearly identified by the PSMEA of the present invention.

The primers used in the present invention were designed to meet the following requirements: 1) the sequence of primer binding site on template should be a type or subtype specific; 2) primers used for PSMEA should exhibit a similar melting temperature. In accordance with the present invention, 11 primers were designed for HCV genotyping with PSMEA (Table 1).

Table 1. Nucleotide sequence of the primers for PSMEA

Primers	Position			Sequence from 5' to 3'
For PCR (universal)				
1st round	-302	to	-278	CTC CCC TGT GAG GAA CTA CTG TCTT (Sense)
	-50	to	-31	CTC GCA AGC ACC CTA TCA GG (Antisense)
2nd round	-204	to	-175	CCA TAG TGG TCT GCG GAA CCG GTG AGT ACAC (Sense)
	-91	to	-74	CCC AAC ACT ACT CGG CTA (Antisense)
For PSMEA				
1AB	-131	to	-111	CTC AAT GCC TGG AGA TTT GGG
1	-176	to	-157	CAC CGG AAT TGC CAG GAC GA
1BR†	-98	to	-78	ACA CTA CTC GGC TAG CAG TCT
2A	-134	to	-114	CCA CTC TAT GCC CGG TCA TTT
2B	-128	to	-108	TAT GTC CGG TCA TTT GGG CAC
2C	-133	to	-113	CAC TCT GTG CCC GGC CAT TTG
3A	-175	to	-157	ACC GGA ATC GCT GGG GTG A
3B	-175	to	-157	ACC GGA ATC GCC GGG ATG A
3R†	-99	to	-79	CAC TAC TCG GCT AGT GAT CTC
5AR†	-236	to	-218	GGG GGT CCT GGA GGC TGT T
6AR†	-145	to	-125	CAT TGA GCG GGT TTG ATC CAA T

† Antisense primer

Fig. 1 illustrates preferred characteristics of primer specific and mispair extension by *pfu*.

In Fig. 1, I illustrates ^{32}P-labeled primer extensions by pfu on template 1b in the presence of dCTP and dGTP (Fig. 1, I-1 and 1, I-2) used as markers for the length of primers and extended products. II illustrates the use of ^{32}P-labeled dNTPs instead of labelled primers for PSMEA under the same reaction conditions, showing the difference in primer extension between genotypes 1a and 1b (Fig. 1, II-1, II-2, II-3 and II-4). III illustrates mispair formation and extension by Taq on template 1a with ^{32}P-labeled primers 1AB and 1BR (Fig. 1, III-1 and III-2). I', II' and III' illustrate the nucleotide sequences extended on templates 1a and 1b. Primer 1AB extended on the antisense strand of templates 1a and 1b. X, XX and XXX represent the sites of nucleotide mismatches that terminated primer extension. → represents a nucleotide at the 3' end of the primer that is complementary to the opposite nucleotide in the template. (A), (C), (G) or (T) denotes the position of the nucleotide when a mispair is produced. -113, -108 and -99 are the nucleotide positions in 5'UR of HCV. Underlined sequence indicate the primer binding sites. The signs in this figure are used for all other figures.

A mismatched pair with nucleotide A at position -99 of the template HCV genotype 1a, that is opposite the first nucleotide to be incorporated at the 3' end of primer could not be produced, and subsequently aborted primer extension by *pfu* in the presence of dCTP and dGTP (Fig. 1, I-1). Under the same reaction conditions, the primer extended on the template of genotype 1b because nucleotide A becomes G at position -99 (Fig. 1, I-2), differentiating between genotypes 1a

and 1b with this single nucleotide variation that is the only difference in the 5' UR between the two genotypes.

FIG. 1 illustrates the alignment of the 5' UR nucleotide sequences of HCV subtypes 1a and 1b, showing the homologous sequence(----) and the difference of the nucleotide at position of -99(A for 1a and G for 1b). The primer extension stopped at position of -99 where a mismatched pair(s) exists (X) in. The arrow (→) represents a nucleotide at 3' end of the primers that is complementary to the opposite nucleotide in the template. The parenthesis ( ) denotes that a nucleotide mispair occurred.

The absence of mispair formation and extension at position -99 is due to *pfu* 3'→5' exonuclease proofreading activity that sharply peaks up at its optimal polymerization temperature, removing the mismatched nucleotide added at the initiation step of primer extension. It is apparently different from the 3'→5' exonuclease proofreading activity of other DNA polymerases such as pol-a and T4 in the primer mispair extension reaction. The mispair at the initiation site of DNA synthesizing can be produced with high frequency by these DNA polymerases in the primer mispair formation and extension reaction (Perinno, F., et al., *Proc. Natl. Acad. Sci. USA.* **86**:8343-8347, 1989), (Reha-Krantz, L.J., Set al., *Proc. Natl. Acad. Sci. USA.* **88**:2417-2421, 1991).

Taq DNA polymerase without 3'→5' exonuclease proofreading activities is used in virtually all PCR based genotyping assays (Okamoto, H., et al., *J. Gen. Virol.* **73**:673, 678,(1992), (Newton, C.R., et al., *Nucl. Acids Res.*, **17**:2503-2516). The unreliability caused by cross reactivity and wrong priming has been a big concern (Lau, J.Y., et al., *J. Infect. Dis.* **171**:

281-289, 1995), and Forns, X., et al., *J. Clin. Microbiology*. **34-10:** 2516-2521, 1996). When *Taq* is used instead of *pfu*, with the other conditions of the assay being the same, the primer 1BR extension took place on template 1a despite a mismatched pair existing at primer extension initiation site (-99) (Fig. 1-III-2). This clearly indicates that the major cause of these nonspecific reactivities is due to the infidelity of *Taq* during the initiation of DNA synthesis (Perinno, F., et al., *Proc. Natl. Acad. Sci. USA*. **86:**8343-8347, 1989).

In addition, since most DNA polymerases including *Taq* and reverse transcriptase of retroviruses exhibit a non-template dependent DNA synthesis activity (i.e. terminal deoxynucleotide transferase activity, TDT), several more nucleotides can be added to the 3' end of the primer (i.e. single strand DNA) (Clark, J.M., *Nucleic Acids Res.* **16:**9677), (Patel, P.H., et al., *Proc. Natl. Acad. Sci. U.S.A.* **91:** 549-553). Thus *Taq* would be able to continue synthesizing DNA by addition of a correct paired nucleotide next to the mispaired nucleotide, in particular to the nucleotide in the A:C mispair that can be formed and extended more efficiently than other mispairs (Perinno, F., et al., *Proc. Natl. Acad. Sci. USA*. **86:**8343-8347, 1989), (Newton, C.R., et al., *Nucl. Acids Res.* **17:**2503-2516). A DNA strand with mismatched nucleotides would be generated and then act as a template that would be subsequently re-amplified, generating a large number of non-specific DNA molecules. Therefore, the nonspecific reaction can not be completely avoided with the methods based on DNA amplification with primer-specific PCR using *Taq* DNA polymerase. In contrast, in PSMEA, only a single primer is used, so that the extended primer cannot be re-amplified, and any mispairs, including A:C

in front of the 3' end of primer could completely stop primer extension. *TLI/Vent* DNA polymerase exhibits 3'→5' proofreading exonuclease activity, however, unlike *pfu*, *TLI/Vent* severely degraded single stranded DNA (i.e. primer) in PSMEA.

In Figs. 1, I-1, primer extension was performed using pfu with labelled primers (1-I), pfu with labelled dNTPs (1-II), and Taq with labelled primers (1-III). The symbol ↑ represents where the nucleotide incorporation starts. The symbol (X) denotes a stop of primer extension caused by the nucleotide mispair(s).

The second feature of specific and mispair extension by *pfu* is that one correct pairing followed by more than one mispair in front of the 3' end of the primer is not sufficient for primer extension (Figs. 2, I-1 and I-2), at least two consecutive correct pairings are required (Fig. 2, I-4 and II-2). This provides a means to identify nucleotide deletion and insertion as well as multiple nucleotide variations or mutations in PSMEA.

In Fig. 2, Primer 6AR-1 could not be extended with template 1a when using either dCTP plus dTTP, or dCTP plus dGTP as substrates due to a CA deletion (..), resulting either in a mispair at nucleotide position -145 in front of the 3' end of primer (I-1) or in only one matched pairing at the position (I-2). Primer 6AR-1 could not be extended on template 6a when using dCTP and dGTP (I-3). However, the primer was extended by three bases with template 6a using dGTP and dTTP which matched the nucleotidees in the CA insertion (CA) in template 6a (I-4). Primer 6AR-2 was extended when using template 6a (II-1 and II-2), but not with template 1a using either dATP, dCTP and dGTP or only dGTP (II-3 and II-4). The symbol ↔ denotes the

removal of the first nucleotide mismatched at the 3' end of the primer.

As seen in Fig. 2, there is a unique CA insertion in the 5' UR of HCV genotype 6a. Use of the CA insertion can differentiate between 6a and other genotypes. In the instant application, the CA insertion is used as an example to show how nucleotide deletion and insertion could be identified. Based on the flanking nucleotide sequence of the CA insertion, two primers were designed. A first primer 6AR-1, located in the region before a CA insertion in HCV genotype 6a was extended only when using the correctly paired dNTPs (i.e. dGTP and dTTP)(Fig. 2, I-4), but not those dNTPs (dCTP and dGTP)(Fig. 2, I-3) that mismatched with the nucleotide A in the CA insertion. The second primer 6AR-2 was designed with the first nucleotide at its 3' end being matched with the first nucleotide A in the CA insertion of template 6a. Thus the primer extended two bases or 11 bases on template 6a, depending on the substrates of dNTPs used (Fig. 2, II-1 and II-2), but not on template 1a due to a CA deletion that results in a 3' mispaired residue being removed after primer binding (Fig. 2, II-3 and II-4).

In accordance with the present invention, multiple nucleotide variation or mutation can be identified. For example, a unique TCA motif that is specific to genotype 3a and 3b could be identified and is used for differentiation between the two genotypes and other genotypes.

Like mispair formation and extension by T4 DNA polymerase (Wilber, J. C., et al., M. S. Reverse transcriptase-PCR for hepatitis C virus RNA, p. 327-331. *In* D. H. Persing, T.F. Smith, F.C. Tenover, and T.J. White (ed.), Diagnostic Molecular Microbiology: Principles and Applications. American Society for

Microbiology, Washington D.C. 1993), any two or more consecutive mispairs could completely terminate primer extension by *pfu* because of its 3'→5' proofreading activity (Figs. 1, II-2 and 2, I-3 and II-2). It was also found that two or more mispairs, but separated with one or two correct base pairs (Figs. 1, II-1 and 2, II-1) also could terminate primer extension by *pfu*. Use of the termination point caused by these mispairs as well as primer specific and mispair extensions on templates by *pfu* provided reliable information on nucleotide sequence in the given region of the 5' UR of HCV.

The highest molecular weight band shown on the sequencing gels represents the longest sequence extended to the termination point that is specific to each of the HCV genotypes. As indicated in Fig. 1, II, for example, the highest band represents the primer 1AB extended to the nucleotide C at nucleotide position -100 before the termination point at position -99 and -98 that would produce two mispairs with the two adjacent nucleotides A in template 1a, while the highest band represents the primer extended to the nucleotide G at -99 before the termination point at position -98 and -96 in template 1b, showing the single nucleotide difference between 1a and 1b. This is another way to identify a single point mutation or variation in PSMEA in accordance with the present invention.

Whatever dNTPs (i.e. one, two or three of the four dNTPs) are chosen, they must follow the "instruction" with the characteristics of primer specific and mispair extension by *pfu*.

The nucleotide incorporation rate of *pfu* is one fifth (1/5) of that of *Taq*. Thus *pfu*-based PCR applications require a minimum extension time of 2.0

minutes/kb. The efficiency of nucleotide incorporation by *pfu* is high enough for PSMEA in which only less than 20 bases of extension are required per cycle in the reaction. Over 50% of the excess primer with less template (molecular ratio of primer and template 10:1) could be extended in a 20 cycle reaction, generating strong signals with either ^{32}P-labelled primer or dNTPs. Thus, PSMEA offers not only an advantage of superior accuracy over current indirect DNA genotyping systems, but an extraordinary sensitivity for detection of mixed infections with different genotypes of HCV.

Direct DNA sequencing is routinely used as the gold standard method for confirmation of the results from other assays (Tuveri, R., et al., *Journal of Medical Virology* **51:** 36-41, 1997), (Forns, X., et al., *J. Clin. Microbiology*. **34-10:** 2516-2521, 1996). In the present application, the sensitivity of PSMEA for identifying mixed infections was evaluated with direct DNA sequencing methods. In an experiment, the cDNAs (PCR products) from genotypes 1b and 2a isolates were mixed in different proportions to mimic HCV mixed infections and heterozygotes. Fig. 3 shows that genotype 2a could be clearly identified by direct DNA sequencing only when it reached to 50% in proportion in the mix. When 2a molecules consisted of less than 25%, only some of nucleotide variation points could be manually recognized, but was not conclusive for the identification of which genotype it was. However, 2a consisting as low as about 3% in the mix was clearly detected by PSMEA, showing approximately 10 times more sensitivity than the direct DNA sequencing system.

To further confirm the sensitivity of PSMEA, three samples identified to contain two or three genotypes by PSMEA were analyzed by direct DNA sequencing (Fig. 3, B) and the reverse hybridization

method. Results reveal that the presence of 2b was confirmed by the direct DNA sequencing method, but not by reverse hybridization method in the sample containing 1a as dominant population.

Fig. 3 presents autoradiography results in A and the date of a computer analysis of automated sequencing in A'. Different proportions of genotypes 1b and 2a: 0% to 100% (I), 50% to 50% (II), 75% to 25% (III), 87.5% to 12.5% (IV), 93.75% to 6.25% (V), and 96.875% to 3.125% (IV) in the mix were analysed with PSMEA (A) and direct DNA sequencing (A'). Two genotypes, 1a and 2b, were identified in a thalassaemia patient sample by PSMEA (B). N in B represents the negative reaction with primer 6AR and the sample from the thalassemia patient.

To evaluate the feasibility of PSMEA for large cohort studies, a total of two hundred and forty five (245) samples from HCV seropositive blood donors and patients with chronic hepatitis were genotyped by this assay. The genotypes determined by PSMEA included 1a, 1b, 2a, 2b, 2c, 3a, 3b, 4, 5a and 6a. The typeable rate of these samples with PSMEA was 95.5%. Eighty (80) of them were also typed with other indirect or direct DNA sequencing genotyping methods. The genotyping results from PSMEA were in 90-100% concordance with that from other genotyping methods including LiPA , RFLP and direct DNA sequencing. Less than 5% of untypeable samples were sequenced, indicating that those isolates being untypeable by PSMEA were either HCV mutants or unclassified genotypes. Results have proven a great utility of PSMEA for large cohort studies on viral genotyping.

PSMEA has been further developed with an automatic and colorimetric format, creating a great capacity to quickly genotype a large number of HCV

isolates not previously possible. Therefore, PSMEA has a great potential for the identification of nucleotide variations and heterozygotes in many areas such as virology, bacteriology, human genetics, epidemiology and legal medicine.

When a type or subtype-specific sequence is available for designing the primer, for example primers 2A and 3A, no cross reativities with other genotypes are observed in PSMEA. Consistent results were obtained with primers 1AB, 2B, 2C, and 3R (Table 2).

Table 2. Identification of HCV genotypes with each of the primers by PSMEA

		HCV Genotypes										
	Primers	1a	1b	2a	2b	2c	3a	3b	4a	4e	5a	6a
Reactivities of	1AB	+	+									+
the Primers	1	+	+						+			
	1BR		+						+	+	+	
	2A			+								
	2B			*	+							
	2C					+						
	3A						+					
	3B							+		+	+	
	3R						+	+				
	5AR										+	
	6AR											+
Criteria for determination of the genotypes by the positivities of the primers		1AB$^+$ 6AR$^-$	1BR$^+$ 1AB$^+$	2A$^+$	2B$^+$ 2A$^+$	2C$^+$	3A$^+$	3B$^+$ 3R$^+$	1+ 1BR$^+$	1BR$^+$ 3B$^+$	5AR$^+$	6AR$^+$

*weak reaction

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Primer 1AB is universal for genotypes 1a and 1b (Fig. 1, III), differentiating the two genotypes from other genotypes except genotype 6a due to the homologous sequence in the region -131 and -99 between genotypes 1a/1b and 6a. Fortunately, a nucleotide A in the unique CA insertion between the nucleotide positions -145 and -144 in the 5' UR of 6a could be used as first paired nucleotide at the 3' end of primer 6AR, thus the primer specifically extended on template 6a, but not other genotypes (Fig. 2, I).

The primer could be bound on other genotypes, but could not be extended due to the CA deletion in these genotypes versus the insertion in 6a 5'UR. It was thus found that, for example, the primer 6AR-1 could not be extended because there a single C at position -145 which could be paired in presence in the presence of dTTP and dGTP, followed by three Ts in the template 1a, in the reaction (Fig. 2, I-1). However, the primer extended on template 6a in the same reaction conditions due to the presence of the two consecutive pairings C:G and A:T at positions between -145 and -144 (Fig. 2, I-4). Accordingly, the primer 6AR2 could be extended longer on template 6a when using three dNTPs (dATP, dGTP and dCTP), showing a stronger signal to differentiate 6a from other genotypes (Fig. 2, II-1). In the same manner, primer 5AR designed with a nucleotide variation at -236 was subtype specific.

As shown in Table 2, primer 3B that was originally designed for identification of genotype 3b exhibited cross reactivity with 4e in PSMEA due to the homologous sequence in the region -175 to -149 chosen between the two genotypes. Thus a small region (-99 to -79) that contains an unique TCA motif in 5'UR of genotypes 3a and 3b was used for designing the primer 3R. Thus by using primers 3A and 3R, genotypes 3a and

3b could be differentiated from other genotypes. Similarly, three genotypes 1a, 1b and 6a could clearly be identified with primer 1AB , 1BR and 6AR that exhibited a cross reactivity with 1AB. A total 11 genotypes including six major genotypes (1a, 1b, 2a, 2b, 3a, 3b) and some of uncommon genotypes (2c, 5a, 6a, 4a and 4e) in Canada could be identified by PSMEA using 11 primers (Table 2).

Extension of the mismatched 3' termini of DNA is a major determinant of the infidelity of the DNA polymerases that have no 3'→5' exonuclease activity, (Pistello, M. et al., *J. Clin. Microbiol.* **32**: 232-234). With the PSMEA of the present invention, by using 3'→5' exonuclease proofreading activity, high detection specificity for nucleotide mutation or variation in a known gene has been achieved with the PSMEA of the present invention. A big advantage of this assay is that a single nucleotide variation, deletion and insertion can be accurately detected. In the genome of many natural virus mutants or drug resistant mutants there may be only a single nucleotide mutation that has potential genetic or clinical significance. For example a substitution C for A at nucleotide position 1814 that destroys the precore initiation codon, will prevent production of HBVeAg. In some of drug resistant mutants, a single nucleotide mutation could cause a failure of an antiviral therapy. PSMEA of the present invention can thus be used for rapid screening of those mutants.

A single point mutation could be associated with genetic disease in human such as a single point mutation resulting in an amino acid substitution (C282Y) in the gene, *HLA-H* for haemochromatosis, which was reported to be involved in iron metabolism disorder. Such single point mutation are frequently

found in viruses. For example, a substitution C for A results in loss of precore initiation codon of hepatitis B virus, preventing e-antigen (HBeAg) synthesis.

The PSMEA of the present invention can be modified so as to replace radioactivity by a detectable label. Such a non-radioactive assay could be in the form of a colorimetric PSMEA in a microtiter-plate format.

The present invention will be more readily understood by referring to the following examples which are given to illustrate the invention rather than to limit its scope.

EXAMPLE I
DETERMINATION OF HCV GENOTYPES

Primer extension reactions contained 20 ng primer, 20-30 ng PCR product, 20 mM of each dCTP and dGTP, 10 mCi of each ^{32}p-labelled dCTP and dGTP, 1.25 units *pfu* DNA polymerase and 10 mL 10X *pfu* reaction buffer (PDI). When 5'-end ^{32}p-labelled primers were used, the ^{32}p-labelled dCTP and dGTP were omitted, and 100 µM of each dCTP and dGTP were used in the reactions. The primer extensions were performed in a reaction volume of 100 mL in a thermocycler (Perkin Elmer, GeneAmp 9600). Twenty (20) cycles consisting of a denaturation step at 94°C for 20 seconds, an annealing step at 64°C for 20 seconds and an extension step at 72°C for 35 seconds were performed. One microliter of the primer extension products was mixed with 1 µL of the sequencing stop solution (Pharmacia Biotech) and electrophoresed on 8% polyacrylamide 8M urea TBE gels for one hour. Extension products were visualized by autoradiography.

For PCR amplification and sequence analysis, viral RNA was isolated from 100 ml of serum by treatment with RNAzol B (Biotecx Laboratories, Houston, Tex.) as previously described in Wilber, J. C., Johnson, P. J., and Urdea, M. S. Reverse transcriptase-PCR for hepatitis C virus RNA, p. 327-331. *In* D. H. Persing, T.F. Smith, F.C. Tenover, and T.J. White (ed.), Diagnostic Molecular Microbiology: Principles and Applications. American Society for Microbiology, Washington D.C. 1993. RT-PCR was performed as described by Bernier et al (Bernier, L., et al., *J. Clin. Microbiol.* **34**:2815-1818), with a set of primers that target highly conserved domains within the 5'-UR. The nucleotides and primers were removed from the PCR products with the QIAquick™ PCR Purification Kit (QIAGEN), using the procedure recommended by the product supplier. These purified PCR products were used for primer extension and automated sequencing analysis.

HCV isolates from patients with HCV infection were typed either by the improved Inno LiPA kit II™ using the procedure provided by the supplier (Innogenetics N.V., Belgium) or by RFLP analysis. For RFLP analysis, HCV genotypes were determined by cleavage of the PCR products with restriction enzymes BstNI, Bsr, Hinfl, Maelll, Haelll, BstUl and ScrFl[19]. Digests were analyzed by gel eletrophoresis and ethidium bromide staining.

EXAMPLE II
PRIMER DESIGN AND PSMEA PROCEDURE

In accordance with a preferred embodiment of the invention, the development of PSMEA is based on a single primer extension in the 5'-UR of the HCV genome using *pfu* DNA polymerase in the presence of only dCTP

and dGTP in the reaction. Thus, under these reaction conditions, primer extension occurs when only the G and/or C nucleotides are added immediately downstream of the 3' end of the primer, since the incorporation of A or T nucleotide would in many instances be prevented by the 3'-5' exonuclease activity of *pfu*. The primers were designed to meet the following requirements:

1. They must have type-specific sequences; and
2. All primers used for PSMEA should exhibit a similar melting temperature.

The nucleotide sequences of the primers used in this study are shown in Table 1. In the PSMEA procedure, the primer extension reactions contained 20 ng primer, 20-30 ng PCR product, 20 mM of each dCTP and dGTP, 10 mCi of each ^{32}P-labelled dCTP and dGTP, 1.25 units *pfu* DNA polymerase and 10 mL 10X *pfu* reaction buffer (Stratagene). When 5'-end ^{32}P-labelled primers were used, the ^{32}P-labelled dCTP and dGTP were omitted, and 100 µM of each dCTP and dGTP were used in the reactions. The primer extensions were performed in a reaction volume of 100 mL in a thermocycler (Perkin Elmer, GeneAmp 9600). Twenty cycles of 94°C for 20 seconds, 64°C for 20 seconds and 72°C for 35 seconds were performed. One microliter of the primer extension products were mixed with 1 µL of the sequencing stop solution (Pharmacia Biotech) and electrophoresed on 8% polyacrylamide 8M urea TBE gels. Extension products were visualized by autoradiography.

EXAMPLE III
PCR AMPLIFICATION AND SEQUENCE ANALYSIS

Viral RNA was isolated from 100 ml of serum by treatment with RNAzol B (Biotecx Laboratories, Houston, Tex.) as previously described in Wilber, J. C., Johnson, P. J., and Urdea, M. S. Reverse transcriptase-

PCR for hepatitis C virus RNA, p. 327-331. *In* D. H. Persing, T.F. Smith, F.C. Tenover, and T.J. White (ed.), Diagnostic Molecular Microbiology: Principles and Applications. American Society for Microbiology, Washington D.C. 1993.. RT-PCR was performed as described by Bernier et al. Bernier, L., et al., *J. Clin. Microbiol.* **34**:2815-1818, with a set of primers (see Table 1) that target highly conserved domains within the 5'-UR. If a second round PCR was necessary to provide sufficient cDNA for PSMEA, a pair of nested primers (sense primer -211 to -192 and antisense primer -91 to -74) was used. The nucleotides and primers were removed from the PCR products with the QIAquick™ PCR Purification Kit (QIAGEN), using the procedure recommended by the manufacturer. These purified PCR products were used for PSMEA and for automated sequencing analysis.

EXAMPLE IV

GENOTYPING OF HCV ISOLATES BY LiPA AND RFLP

HCV isolates from patients with HCV infection were typed either by the improved Inno LiPA kit II™ using the procedure provided by the supplier (Innogenetics N.V., Belgium) or by RFLP analysis. For RFLP analysis, HCV genotypes were determined by cleavage of the PCR products with restriction enzymes BstNI, Bsr, Hinf1, Maell1, Haell1, BstU1 and ScrF1, (Andonov, A., et al., *J. Clin. Microbiol.* **32.** 2031-2034, 1994). Digests were analyzed by gel electrophoresis and ethidium bromide staining.

EXAMPLE V

The accuracy and reliability of PSMEA

To evaluate the accuracy and reliability of PSMEA, 51 HCV isolates from HCV infected individuals in Montreal, Canada, typed by RFLP analysis in the 5' UR

(Andonov, A., et al., *J. Clin. Microbiol.* **32.** 2031-2034, 1994) were analyzed with PSMEA in a double blind study. The subtypes 2a and 2c were grouped together when RFLP was used for genotyping of the 51 isolates, since some 2c variants share common ScrFI cleavage sites with 2a variants.

A primer designed with sequence -133 to -113 from this isolate has shown no cross reactivity with other types described in the present study (Table 2), suggesting that most isolates determined as 2a/2c by RFLP have a typical 2a specific sequence, and that the primer 2A can discriminate the majority of subtype 2a from subtype 2c. The results from the analysis of these 51 isolates indicate that 100% of types and subtypes for the isolates determined by PSMEA were matched with the types and subtypes identified with RFLP. In these isolates, there were 14-1a, 14-1b, 5-2a, 5-2b, 1-2c, 10-3a, 1-3b, and 1-6a. LiPA was reported to reliably type the most common genotypes, including some subtypes (Simmonds, P., Variability of Hepatitis C. Virus. *Hepatology* **21:** 570-582, 1995). Fifteen (15) isolates were typed by LiPA and evaluated with PSMEA in accordance with the present invention. They include 5-1a, 6-1b, 1-2a, 1-2b, 1-3a, 1-3b. The results from PSMEA showed a 100% agreement with that from LiPA.

A group of five (5) HCV isolates that failed to be typed or subtyped by other methods were clearly identified as 1a or 1b by PSMEA. The results were confirmed by direct DNA sequencing using their PCR products from the 5' UR region (-211 to -71) (Fig. 4), suggesting that results from PSMEA is reliable.

In the present application, the majority of the samples were typed with PSMEA using non-labelled primers and ^{32}p-labelled dNTPs as shown in Fig. 6.

Results obtained using ^{32}p-labelled dNTPs with unlabelled primers showed the typical patterns of the primer extensions as seen in the results from the reaction with ^{32}p-labelled primers and non-labelled dNTPs.

Accordingly the new genotyping assay, primer specific and mispair extension assay (PSMEA) of the present invention was used to genotype HCV and to detect mixed infections. A total of one hundred and forty six (146) HCV isolates were typed and analyzed with PSMEA, showing that nine of 110 isolates (8.2%) from HCV positive blood donors and six of 36 isolates (16.7%) from thalassaemia patients were found to contain more than one genotypes. The results were confirmed and compared with other current assays including direct DNA sequencing and line probe assay (LiPA). PSMEA of the present invention was found to be more reliable than other assays in detecting mixed infection.

EXAMPLE VI

Feasibility of PSMEA for large cohort studies

Some genotypes including 1a, 1b, 2a, and 2b show a broad geographical distribution, and the infection frequency of subtypes 1a and 1b can be over 50% in blood donors and patients with chronic hepatitis in the United States (Lau, J.Y., et al., *J. Infect. Dis.* **171:** 281-289, 1995), Canada (Bernier, L., et al., *J. Clin. Microbiol.* **34:**2815-1818), (Andonov, A., et al., *J. Clin. Microbiol.* **32.** 2031-2034, 1994), and most European countries (Simmonds, P., *Hepatology* **21:** 570-582. 1995), (Stuyver, L., Rosseau, et al., *J. Clin. Microbiol.* **34:**2259-2266, 1996). Other genotypes , such as 3a and 3b are less common than 1a and 1b in those countries. However, 3a seems to be quite frequently

found in Canada. Genotypes 4, 5a and 6a are only found in specific geographical regions in the Middle East, South Africa and Hong Kong respectively, but were also infrequently found in some areas of Canada. It is apparent that for each region a strategy for genotyping a large number of HCV isolates by PSMEA has to be designed, based on the genotype distribution and infection frequency with the population. For example, in Canada, over 60% of HCV isolates are genotypes 1a, 1b, which can be identified by primers 1AB, 1BR. However, since genotype 6a was frequently found in some areas of Canada and primer 1AB was cross reacted with 6a, thus all isolates diagnosed as 1a should be retested with primer 6AR for screening of genotype 6a in the first round of testing with PSMEA. The non-typeable isolates using the three primers should be retested with primers 2A and 2B. Thus genotypes 2a and 2b (over 15% of total isolates) can be determined in the second round of testing. The remaining non-typeable isolates should be screened with primers 3A, 3B and 3R. Genotypes 3a and 3b (over 15% of total isolates) can be identified in the third round of testing. After the three round testing, the rest of isolates (less than 10% of total isolates) that cannot be typed by these primers would include some subtypes of type 4, subtype 5a, subtype 2c or other genotypes. Table 3 identifies the infection frequencies of the major HCV genotypes identified by PSMEA, indicating that it is practical and feasible for genotyping a large number of isolates for epidemioloy and clinical studies.

All current genotyping assays such as for HCV, including direct DNA sequencing, are not suitable for detection of mixed infections because they are designed for detection of the population-dominant genotype. As

indicated in Table 3, HCV mixed infection rate was higher than expected. A reliable detection of HCV mixed infections by PSMEA in different populations with HCV infection is thus reported.

While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth, and as follows in the scope of the appended claims.

WHAT IS CLAIMED IS:

1. A primer-specific and mispair extension assay for the determination of genotype, said assay comprising the steps of:

a) extending a DNA sequence amplified and detection of low level mixed genotype injectius or heterozygotes, from a patient sample with *PFU* DNA polymerase using a primer specific for a genotype to be determined and an incomplete set of dNTPs, under suitable conditions for obtaining extension of the primer, whereby at least one of the primer or one of the dNTPs is labelled;

b) separating the amplified DNA sequences obtained in step a);and

c) detecting the separated amplified DNA sequences; and

d) comparing the amplified DNA sequences with known DNA sequences of various genotypes for determining the genotype of the DNA sequences amplified.

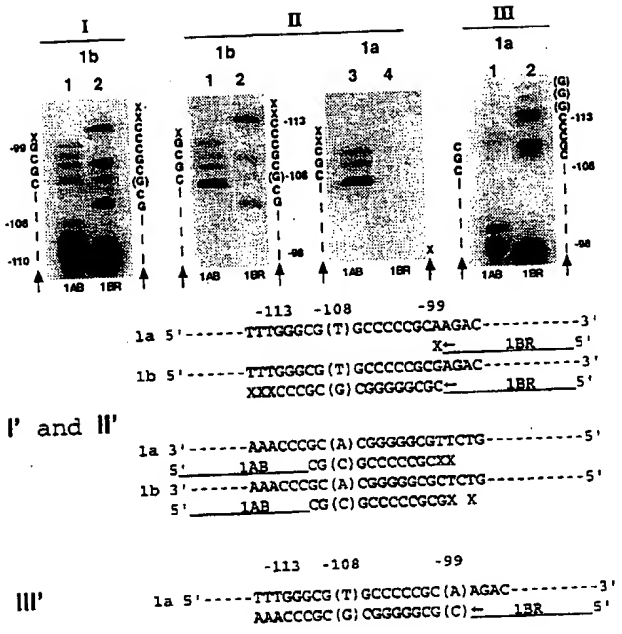
2. The assay of claim 1, wherein the primer is end-labelled with a radioactive label.

3. The assay of claim 1, wherein one of the dNTPs is labelled with a radioactive label.

4. The assay of claim 1, wherein the primer is end-labelled with a fluorescent label.

5. The assay of claim 1, wherein steps a), b), c), and d) are automated.

6. The assay of claim 1, wherein step c) further comprises the step of sequencing the separated amplified DNA sequences.


I
II
III
1b
1a
1 2
3 4
-113
-108
-99
1AB
1BR
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X← 1BR 5'
1b 5'------TTTGGGCG(T)GCCCCCGCGAGAC----------3'
XXXCCCGC(G)CGGGGGCGC← 1BR 5'
I' and II'
la 3'------AAACCCGC(A)CGGGGGCGTTCTG----------5'
5' 1AB CG(C)GCCCCCGCXX
1b 3'------AAACCCGC(A)CGGGGGCGCTCTG----------5'
5' 1AB CG(C)GCCCCCGCGX X
III'
la 5'-----TTTGGGCG(T)GCCCCCGC(A)AGAC---------3'
AAACCCGC(G)CGGGGGCG(C)← 1BR 5'

Fig. 1

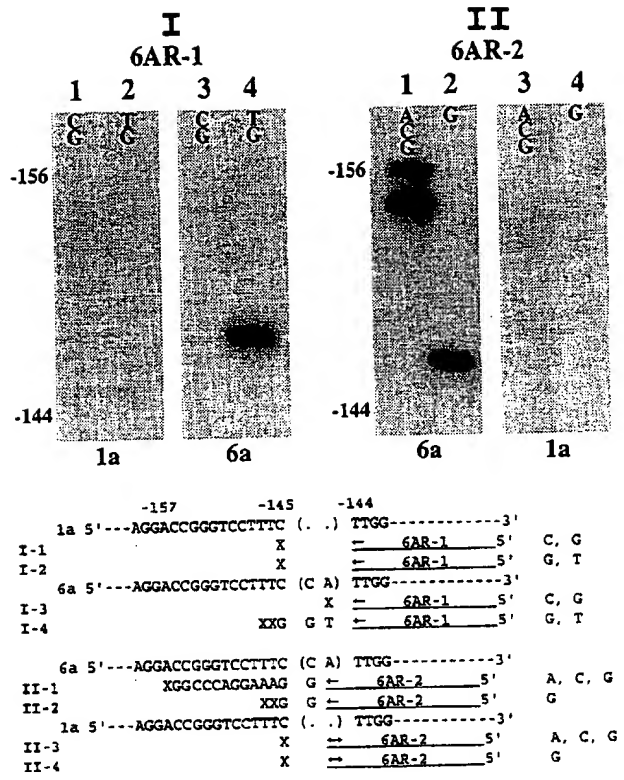
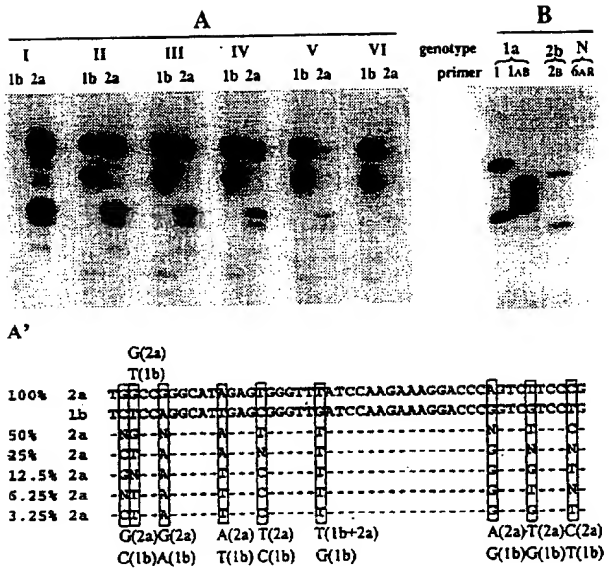

I
6AR-1
1 2 3 4
-156
-144
1a
6a
II
6AR-2
1 2 3 4
-156
-144
6a
1a
-157
-145
-144
1a 5'---AGGACCGGGTCCTTTC (. .) TTGG----------3'
I-1 X ← 6AR-1 5' C, G
I-2 X ← 6AR-1 5' G, T
6a 5'---AGGACCGGGTCCTTTC (C A) TTGG----------3'
I-3 X ← 6AR-1 5' C, G
I-4 XXG G T ← 6AR-1 5' G, T
6a 5'---AGGACCGGGTCCTTTC (C A) TTGG----------3'
II-1 XGGCCCAGGAAAG G ← 6AR-2 5' A, C, G
II-2 XXG G ← 6AR-2 5' G
1a 5'---AGGACCGGGTCCTTTC (. .) TTGG----------3'
II-3 X → 6AR-2 5' A, C, G
II-4 X → 6AR-2 5' G

Fig. 2


A
B
I
II
III
IV
V
VI
1b 2a
1b 2a
1b 2a
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1b 2a
genotype
primer
1a
1AB
2b
2B
N
6AR
A'
G(2a)
T(1b)
100%
2a
1b
50%
2a
25%
2a
12.5%
2a
6.25%
2a
3.25%
2a
G(2a)G(2a)
C(1b)A(1b)
A(2a)
T(1b)
T(2a)
C(1b)
T(1b+2a)
G(1b)
A(2a)T(2a)C(2a)
G(1b)G(1b)T(1b)

Fig. 3